A comparison of methane emissions from beef cattle measured using methane hoods with those measured using respiration chambers

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Development and use of microsatellite markers to study diversity, reproduction and population genetic structure of the cereal pathogen *Ramularia collo-cygni*.

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Ramularia collo-cygni (*Rcc*), Ramularia Leaf Spot (RLS), microsatellite (SSR), growth stage (GS), Quinone outside Inhibitors (QoIs)

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Abstract

Ramularia collo-cygni (*Rcc*) is a major pathogen of barley that causes economically serious yield losses. Disease epidemics during the growing season are mainly propagated by asexual air-borne spores of *Rcc*, but it is thought that *Rcc* undergoes sexual reproduction during its life cycle and may also disperse by means of sexual ascospores. To obtain population genetic information from which to infer the extent of sexual reproduction and local genotype dispersal in *Rcc*, and by implication the pathogen's ability to adapt to fungicides and resistant cultivars, we developed ten polymorphic microsatellite markers, for which primers are presented. We used these markers to analyse the population genetic structure of this cereal pathogen in two geographically distant populations from the Czech Republic (n =30) and the United Kingdom (n =60) that had been sampled in a spatially explicit manner. Genetic diversity at the microsatellite loci was substantial, H_t =0.392 and H_t =0.411 in the Czech and UK populations respectively, and the populations were moderately differentiated at these loci (Θ =0.111, P <0.01). In both populations the multilocus genotypic diversity was very high (one clonal pair per population, resulting in >96% unique genotypes in each of the populations) and there was a lack of linkage disequilibrium among loci, strongly suggesting that sexual reproduction is an important component of the life cycle of *Rcc*. In an analysis of spatial genetic structure, kinship coefficients in all distance classes were very low (-0.0533 to 0.0142 in the Czech and -0.0268 to 0.0042 in the Scottish population) and non-significant (P >0.05) indicating lack of subpopulation structuring at the field scale and implying extensive dissemination of spores. These results suggest that *Rcc* possesses a high evolutionary potential for developing resistance to fungicides and overcoming host

resistance genes, and argue for the development of an integrated disease management system that does not rely solely on fungicide applications.

Keywords: *Ramularia collo-cygni*, microsatellites, population structure, genetic diversity, evolutionary potential

1. Introduction

The last four decades have brought rapid intensification of agriculture, which has led to a twofold increase in food production. This has been made possible through a combination of several factors, including cultivation of highly yielding varieties, soil fertilization and irrigation and effective control of crop pathogens, insects and weeds with chemical products (Oerke & Dehne, 2004; Tilman, 1999). Effective chemical control of biotic stresses has significantly reduced the yield losses in crops (Oerke & Dehne, 2004) whereas the narrow genetic variability of crop species has created a suitable environment for the adaptation and emergence of new crop diseases (Stukenbrock & McDonald, 2008). Currently plant protection is challenged both by the evolution of pathogens that overcome host resistance genes and by the evolution of losses of sensitivity mutants to commonly used chemical control products (Hollomon & Brent, 2009).

To predict the capacity of pathogen populations to adapt to changing environments, including such control measures as fungicide application and resistant cultivars, it is essential to understand their evolutionary potential (McDonald & Linde, 2002). A population genetic approach has been successfully used to assess the evolutionary potential of pathogens such as *Zymoseptoria tritici* (Banke & McDonald, 2005; Linde *et al.*, 2002; Medini & Hatnza, 2008), *Melampsora larici-populina* (Barres *et al.*, 2012; Xhaard *et al.*, 2011) and *Rhynchosporium commune* (McDonald *et al.*,

1999; Zaffarano *et al.*, 2009). The important parameters from which evolutionary potential can be inferred are the genetic diversity of populations, the reproduction system controlling the ability to recombine genetic variants, and the dispersal ability of the pathogens. In the context of the evolution of fungicide resistance, the greater the genetic diversity of populations, the greater the standing genetic variation from which resistant variants may be selected. In terms of the mating system, populations undergoing sexual or mixed reproduction are more likely to develop resistance than population reproducing only clonally because sex facilitates recombination and allows new resistant mutations to become associated with genotypes at other loci that confer greatest fitness. Furthermore in sexually reproducing populations such resistant genotypes of high fitness can then be dispersed effectively by sexual as well as asexual spores, and the resistance phenotype can be rapidly established in the population. In asexually reproducing populations parasexualism can generate some recombination, but the process is much less effective than that involving sexual reproduction and does not generate sexual spores for dispersal (Barrett *et al.*, 2008; McDonald & Linde, 2002).

A very efficient way of establishing the genetic diversity of pathogen populations, and inferring their mating system and dispersal capacity is to analyse variation at selectively neutral molecular marker loci in spatially defined samples from natural populations. In this study we develop and used microsatellite (SSR) genetic markers to explore the genetic diversity, reproductive system and genetic structure of the emergent fungal pathogen of barley *Ramularia collo-cygni* (*Rcc*). We use these data to infer the potential of populations of this pathogen to evolve in response to management changes such as fungicide application and growth of resistant cultivars.

Ramularia collo-cygni is an ascomycete fungus causing Ramularia Leaf Spot (RLS) disease in both spring and winter barley (*Hordeum vulgare*), (Huss, 2004; Oxley *et al.*, 2002). It was reported for the first time in 1893 in Northern Italy (Cavara, 1893). However it is only in the last 20 years that the disease has been associated with serious economic losses in countries across Europe, as well as other continents worldwide (Sachs *et al.*, 1998; Sachs, 2006). The significant inoculum sources of the disease are winter barley (Frei *et al.*, 2007) and infected barley seed (Havis *et al.*, 2006; Havis *et al.*, 2014; Matusinsky *et al.*, 2011). Additionally some volunteers, crop debris and other grasses are considered to act as possible inoculum sources (Frei *et al.*, 2007; Huss, 2004; Salamati & Reitan, 2007; Walters *et al.*, 2008). Spores of *Rcc* are air-borne and sporulation events have been shown to occur after a certain amount of leaf wetness over the season (Frei *et al.*, 2007; Huss, 2004; Oxley & Havis, 2010; Salamati & Reitan, 2007). To the present moment the teleomorph stage of the pathogen has not been observed. As breeding for resistance in barley has not so far been successful (Havis *et al.*, 2012; Matusinsky *et al.*, 2013; Oxley & Havis, 2010), RLS in barley is currently controlled by fungicide applications (HGCA, 2013). Therefore it is essential to establish the evolutionary potential of the pathogen to better assess the risk of fungicide resistance development and the potential success of future resistance breeding programmes.

Previous studies of population genetic structure in *Rcc* have used dominant, biallelic, AFLP markers to investigate its genetic and clonal diversity and the distribution of this diversity among countries, regions and individual fields (Hjortshoj *et al.*, 2013; Leisova-Svobodova *et al.*, 2012). While the AFLP technique used in these studies generates many markers in a cost effective manner, it suffers from the limitations that individual loci are anonymous, relies on the assumption that

fragments of identical size represent homologous regions of DNA, and generates results that are difficult to reproduce between laboratories. These limitations can be overcome by developing microsatellite markers that target particular regions of DNA, generate loci with many alleles possessing high information content, and produce results that can readily be compared between research groups (Jarne & Lagoda, 1996; Oliveira *et al.*, 2006; Schlotterer, 2000; Selkoe & Toonen, 2006; Sunnucks, 2000).

In this study we develop a set of ten primer pairs for SSR loci that can be applied reproducibly to investigate the population genetics of *Rcc*. We use these markers to measure detailed genetic diversity and genetic structuring of *Rcc* within two fields that have been sampled in a spatially explicit manner. Our aim is not only to make inferences about the role of sexual reproduction in *Rcc*, but also to explore the effectiveness of spore dispersal within populations by determining the extent of spatial clustering of genotypes within each field.

2. Materials and methods

2.1. Microsatellite primers development

Microsatellite primers were derived using data from a Scottish Government funded *Rcc* whole genome sequencing project at SRUC (McGrann *et al.*, unpublished data; genome browser: http://ramularia.org/jbrowse). Microsatellites were predicted using MISA (Thiel *et al.*, 2003) using the standard misa.ini file (i.e. definition: unit_size, min_repeats: 1-10 2-6 3-5 4-5 5-5 6-5; interruptions: max_difference_between_2_SSRs: 100). Both the genomic and transcriptomic sequences were used to predict microsatellites loci of different repeat motifs (di-, tri-tetra-, penta- and combined motifs). Perl scripts linked with the Primer3 program

149 (Untergasser *et al.*, 2012) were subsequently used to design primers from the
150 flanking regions of the microsatellites. For the purpose of this study we chose ten
151 sets of primers (Eurogentec) amplifying pentanucleotide microsatellite loci in the
152 genomic sequence of *Rcc* (Table 1) and we tested them across ten *Rcc* strains
153 originating from a SRUC worldwide collection. Loci were chosen from different
154 contigs and with non-overlapping allele lengths to facilitate PCR multiplexing of loci
155 and increase the chance of their location on different chromosomes. Each of the F
156 primers was tailed at the 5' end with M13 universal primer and the M13 primer was
157 labelled with 6-FAM dye at the 5' end as previously described by Schuelke (2000).

158 **2.2. Study populations and sampling strategies**

159 We analysed 90 isolates of *Rcc* collected from spring and winter barley
160 cultivars in two European countries, the Czech Republic and the United Kingdom.
161 Isolates from the Czech Republic (n =30) were supplied by Pavel Matusinsky from
162 Agrotest Fito, Ltd, Kromeriz. Leaf samples (F-1 or flag leaves) were collected at
163 Krenovice (49°19'30.986"N, 17°15'44.491"E) in June 2012 from a highly infected
164 winter barley cultivar Traminer at growth stage (GS) 85 (Zadoks *et al.*, 1974). In total
165 100 isolates from two transect lines separated by 16 meters, were obtained. Along
166 each of two transect lines separated by 16 meters, a total of 50 locations were
167 sampled at intervals of a meter. We used thirty randomly chosen isolates from this
168 collection in this study (Figure A. 1).

169 In the UK we sampled a commercial crop of the spring barley cultivar Waggon
170 at the end of the growing season (GS 85) at Bush Estate (55°52'15.449"N,
171 3°12'9.787"W) in Boghall near Edinburgh in August 2012 following slightly modified
172 procedure of McDonald *et al.* (1999). Sixty isolates derived from two transect lines,
173 20 meters apart, were amassed (n =60). On each line three circles one meter in

diameter separated by 20 meters were sampled. At each location ten F-1 leaves from different plants around the circle were removed (Figure A. 2). This collection procedure enabled us to obtain hierarchically sampled isolates at the subpopulation level, defining each circle as one subpopulation.

2.3. DNA extraction

Single spore isolates of *Rcc* were obtained from sampled leaves and cultivated on PDA media amended with streptomycin 5 µg/ml and/or kanamycin 50 µg/ml in a phytotron in the dark, at 15°C. After three to four weeks fungal material was collected in Eppendorf tubes, freeze dried overnight and ground to a fine powder in a tissue lyser (Tissue Lyser LT, Qiagen). DNA of *Rcc* isolates was extracted using Illustra Nucleon PhytoPure Genomic DNA Extraction Kit (GE Healthcare Life Sciences), according to the manufacturer's instructions. If high protein content was observed, a second step of DNA purification using a mixture of chloroform-phenol-isoamyl alcohol (Sigma-Aldrich) was performed.

2.4. Multiplex PCR

SSR primers were grouped into two mixes for multiplex PCR: MixI: SSR2, SSR4, SSR7, SSR11 and MixII: SSR1, SSR3, SSR5, SSR6, SSR8, SSR12. Amplification was carried out using the Multiplex PCR Kit (Qiagen). Each of the amplification reactions was composed of 1x Master Mix, 0.2 µM of a final concentration of each R primer and M13 primer and 0.5 µM of each F primer, 12.5 ng of DNA template and RNase free water to a total volume of 25 µl (Qiagen). The thermocycler conditions were as follows: initial denaturation at 95°C for 15 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 1.5 minutes, extension at 72°C for one minute and final extension at 60°C for 30 minutes.

2.5. Microsatellite analysis

PCR products were run on an ABI 3730 automated sequencer at DBS Genomics (Durham, UK). The sizes of DNA fragments were scored and alleles differing in size by a single base (and in very few cases by two) were manually binned for genetic analysis. Genotype input files were formed with CREATE v1.37 (Coombs *et al.*, 2008). To identify clonal genotypes and calculate the probability of genotype identity (Woods *et al.*, 1999) GIMLET software v1.3.3 was used (Valiere, 2002). After identification and removal of clonal replicates, mean gene diversities for each locus (H_{sk}), overall gene diversity (H_t), allelic richness (A), linkage disequilibrium and genetic differentiation were calculated with FSTAT v2.9.3.2 (Goudet, 1995; Goudet, 2002). Linkage disequilibrium was estimated as the correlation between the pairs of loci (Weir, 1996). H_{sk} and H_t were estimated using Nei's (1987) unbiased and unweighted estimators respectively and Weir and Cockerham's (1984) estimator of the F_{st} (Θ) was used to measure genetic differentiation among populations. Significance of Θ was based on 15000 bootstraps over loci while significance level for genotypic disequilibrium was based on 900 permutations with P values adjusted for 5% nominal level to P =0.001. The spatial structure of *Rcc* populations was analysed using SPAGeDi v1.4 software (Hardy & Vekemans, 2002) based on pairwise kinship coefficients for co-dominant markers according to Loiselle *et al.* (1995). The data in the Czech population was divided into five pairwise distance classes, and in the Scottish population into four. Significance of the substructuring was based on 1000 random permutations.

3. Results

3.1. Genetic diversity

All ten pentanucleotide SSR loci chosen showed polymorphism in the total *Rcc* sample. Within individual populations 90% of loci were polymorphic with monomorphism at SSR5 and SSR11 in the Czech and Scottish populations respectively. The two populations showed similar level of genetic diversity across ten SSR loci (Table 2). The mean numbers of alleles per locus were A =3.2 and A =3.7 and mean calculated gene diversities over all loci were H_t =0.392 and H_t =0.411 in the Czech and Scottish populations respectively.

3.2. Genotypic diversity and reproduction system

The genotypic diversity in the tested populations was very high and the majority of the isolates within and between tested populations had unique genotypes. Only one clonal pair was identified in each population; isolates CZ12Rcc031 and CZ12Rcc039 in the Czech population (Figure A. 1) and isolates GBS12Rcc090 (b1) and GBS12Rcc121 (e2) in the Scottish population (Figure A. 2). In the Czech population these clonal isolates were separated by 25.6 meters and in the Scottish population one clonal pair was identified among strains sampled approximately 21 meters apart. The probability that these two pairs of identical genotypes have been formed independently via sexual reproduction was very low in both the Czech (P =1.70 x 10-7) and the Scottish (P =5.06 x 10-5) populations. The two populations shared only one multilocus genotype in common, the remainder of the genotypes being unique. The analysis of linkage disequilibrium demonstrated that there was no reason to reject the null hypothesis of random association between alleles at different loci (P >0.001) in either populations.

3.3. Population differentiation and isolation by distance

Significant moderate genetic differentiation (Θ =0.111, P <0.01) was found between the two populations. To determine whether there was any genetic structuring within the field populations, spatial genetic analysis based on pairwise kinship coefficients (Loiselle *et al*., 1995) was performed on both the Czech and the Scottish populations (Figure 1, Figure 2). The kinship coefficients in each distance class were quite low and not significantly different from zero (P >0.05), ranging from -0.0533 to 0.0142 in the Czech population and from -0.0268 to 0.0042 in the Scottish population of *Rcc*, indicating no spatial genetic structure within either population. Additionally within the Scottish population there was no detectable differentiation among the six subpopulations that had been sampled (Θ =0.013, P >0.05).

4. Discussion

The microsatellite primer pairs designed in this study from *Rcc* genome sequence information (McGrann *et al.*, unpublished data) successfully amplified ten polymorphic microsatellite loci. Loci were sufficiently variable to allow detailed studies of clonal structure, genotypic diversity, linkage disequilibrium and spatial genetic structure. These markers are likely to be a valuable resource for further population genetic studies of *Rcc*, yielding data that can be readily shared and directly compared among different research groups. The benefits of using highly variable and reproducible microsatellite markers have already been well illustrated in comparative studies of a range of other pathogenic fungi (Barnes *et al.*, 2008; Barres *et al.*, 2012; Dilmaghani *et al.*, 2012; Gurung *et al.*, 2011; Gurung *et al.*, 2013; Linde *et al.*, 2005; Rieux *et al.*, 2013; Stefansson *et al.*, 2012; Tomsovsky *et al.*, 2013).

4.1. The role of sexual reproduction in *Rcc* populations

The most striking result to emerge from the microsatellite analysis of genotypic diversity is that in both populations all except one of the multilocus genotypes found was sampled only once. Populations comprise a very diverse collection of genotypes, and no single genotype or small collection of genotypes dominates the population. This occurs despite the fact that in both populations sampling took place at the end of the season after a period of extensive asexual reproduction and spread. The result implies that differences in fitness among genotypes are not large, for if this were the case the population would quickly be dominated by the fitter genotypes. Previous studies using AFLP markers have also found very high genotypic diversity within a range of *Rcc* populations across Europe (Hjortshoj *et al.*, 2013; Leisova-Svobodova *et al.*, 2012). The situation contrasts markedly with that found in other pathogens such as *Phytophthora infestans* where single genotypes may increase rapidly in frequency and come to dominate large geographic areas (Cooke *et al.*, 2012). Although our findings of high genotypic diversity agree with the results of previous studies on *Rcc*, we failed to detect any linkage disequilibrium among the microsatellite loci scored, a result that is at variance with previous reports of linkage disequilibrium among AFLP loci in *Rcc* populations (Hjortshoj *et al.*, 2013; Leisova-Svobodova *et al.*, 2012). Linkage disequilibrium is likely to be generated in *Rcc* populations by differential asexual reproduction of multilocus genotypes through the growing season (Frei *et al.*, 2007; Huss, 2004; Salamati & Reitan, 2007). If there is sexual reproduction, this linkage disequilibrium will be broken down at a rate that is proportional to the recombination fraction between the loci.

A number of reasons could account for discrepancies in the level of linkage disequilibrium found here and in the previous studies. The first relates to the nature of

the markers studied. In our study markers were chosen from different linkage groups, thus maximising the rate of decay of linkage disequilibrium following sexual reproduction. In contrast the genomic location of AFLP markers is unknown, and if pairs of markers were tightly linked this would reduce the effect of sexual reproduction in reducing linkage disequilibrium.

The second reason for a difference in the result is that patterns of selection could be different between the studies, with stronger differential selection among genotypes in populations previously studied. The third possibility is that sexual reproduction may have been more frequent in the populations studied here than in previous studies, leading to a reduction in the level of linkage disequilibrium. Finally it is also possible that we failed to detect linkage disequilibrium due to relatively small sample sizes used in this study (n =30 in Czech Republic and n =60 in Scotland). It was shown previously that in most of the cases quite large sample sizes were required to detect linkage disequilibrium in the populations (Brown, 1975).

Whatever the reason for the observed discrepancies, it should be noted that even when linkage disequilibrium is detected its level is low, and we can conclude that sexual reproduction has or is occurring in *Rcc*, at least to the extent required to prevent the build-up of extensive linkage disequilibrium. We cannot however exclude the possibility that currently the role of sex in *Rcc* is minor but large population size and possibly more frequent sexual reproduction in the past has generated the observed results. A follow on project is underway to directly investigate the reproductive biology and crossing potential of *Rcc*. Previous data has suggested that a cryptic sexual stage does exist (Kaczmarek, personal communication). Similar population genetics studies to ours have provided evidence for sexual reproduction in

323 the causal agent of barley scald, *Rhynchosporium commune*, which was originally
324 thought to reproduce only asexually (McDonald *et al.*, 1999).

325 **4.2. Effectiveness of spore dispersal within populations**

326 We found no isolation by distance and significant amounts of gene flow within
327 both field populations, indicating extensive spore dispersal of *Rcc* across the field
328 (Barrett *et al.*, 2008; Biek & Real, 2010). Individuals showed a low genetic
329 relatedness even in the shortest distance class (6.6 meters for the Czech population
330 and 8.7 meters for Scottish population). In the Scottish population of *Rcc*, clonal
331 genotypes were separated by around 21 meters and in the Czech population by
332 around 25.6 meters. Thus the distance that the asexual spores of *Rcc* can be
333 disseminated is at least this distance. However given the limited number of samples
334 analysed in this study as well as the relatively small spatial scale of this experiment,
335 potential dissemination distances for spores could easily be longer.

336 A similar lack of spatial structure was previously described for *Mycosphaerella*
337 *fijiensis*, a causal agent of banana and plantain (Rieux *et al.*, 2013), whose
338 ascospores were shown to have the capability for long-distance dispersal (Burt *et al.*,
339 1998). In contrast in *Dothistroma septosporum* (an ascomycete pathogen closely
340 related to *Rcc*) genetic structuring at the local scale, up to a distance of 8-12 meters
341 was found (Kraj & Kowalski, 2013). The asexual conidia of the pathogen, that are
342 rain-splashed over short distances, were shown to be the most important source of
343 infection over the season and the sexual stage of the fungus is thought to be less
344 frequent and also to occur for a shorter period of time (Gibson, 1972; Karadzic, 1989;
345 Peterson, 1973). Similar results have also been found in the ascomycete canker
346 pathogen of pine, *Gremeniella abietina* (Wang *et al.*, 1997). Thus is it very likely that
347 the extensive gene flow and the lack of isolation by distance within field populations

of *Rcc* result from the extensive dissemination of air-borne asexual spores over the epidemic season coupled with possible long distance dispersal of ascospores following sexual reproduction.

4.3. Genetic differentiation and the mode of possible dispersal between *Rcc* populations

We found moderate genetic differentiation (Θ =0.111, P <0.01) between the two sampled locations in the Czech Republic and Scotland. A previous study using AFLP markers described genetic differentiation of F_{st} =0.123 (P <0.001) between 19 locality population in Central Europe (Leisova-Svobodova *et al.*, 2012). In Northern Europe the fixation index between two distinct populations from Denmark and Scotland based on AFLP markers was G_{st} =0.031 (P =0.01) suggesting that the populations were genetically fairly similar. Higher differences in allele frequencies were found among two Danish subpopulations (G_{st} =0.135, P =0.001), however no significant differentiation was indicated among Scottish subpopulations (G_{st} =0.042, P =0.096), (Hjortshoj *et al.*, 2013). Furthermore both of the studies indicated that most of the genetic variation was distributed on a small scale (within field, plots, and localities) rather than over larger geographical areas (Hjortshoj *et al.*, 2013; Leisova-Svobodova *et al.*, 2012), which confirms the results presented here using SSR markers.

The observed genetic differentiation between the two studied populations of *Rcc* from the Czech Republic and Scotland suggests that these populations either had the same origin but due to the occurrence of genetic drift in each of the populations, genetic discontinuity was observed, or that gene flow occurs through either long-distance dispersal of pathogen spores for several hundred kilometres or through the transport of infected barley seeds. *Ramularia collo-cygni* is a seed-borne

pathogen and could be introduced to new territories through the transport of infected seed (Havis *et al.*, 2006; Havis *et al.*, 2014; Matusinsky *et al.*, 2011), a likely scenario which has been previously described by Hjortshoj *et al.* (2013) and Leisova-Svobodova *et al.* (2012) and which was shown to be responsible for migration events in another barley pathogen *Rhynchosporium commune* (Linde *et al.*, 2009).

On the other hand the asexual spores of *Rcc* are thought to be air-borne and already Huss (2004) proposed that they could be disseminated to new locations with wind currents which could contribute to recent disease establishment events. Thus it is possible that gene flow among *Rcc* populations occurs through a combination of long-distance spore dispersal and the transport of infected seeds, which brings a need for an effective seed control method especially in territories where the pathogen is currently absent. Furthermore, spores of the pathogen are likely to be dispersed over a long distance which indicates the threat of a rapid spread of both fungicide resistance alleles and potential resistance genes bred into cultivars within field populations, as well as between more distant localities. However it is also possible that there may have been little time for differentiation to have built up among populations following the recent dispersal of the species to diverse areas through seed movement or spore dispersal and because of that moderate or no differentiation between population is observed in this study as well as in the previous ones (Hjortshoj *et al.*, 2013; Leisova-Svobodova *et al.*, 2012).

4.4. Conclusions

From an evolutionary perspective pathogen populations that are characterised by a large population size, a high level of gene flow, a high mutation rate and that undergo both sexual and asexual reproduction cycles over the season are considered to be the most risky in terms of their adaptive potential for overcoming

resistance to such control measures as fungicide applications and cultivars resistant to the pathogen (McDonald & Linde, 2002). The genetic parameters that we estimated in this study suggest that populations of *Rcc* possess a great adaptive potential and pathogen associated risk from the perspective of biological characteristics should be considered as high. To date, *Rcc* has only developed wide spread resistance to the Quinone outside Inhibitors (QoIs) group of fungicides (Fountaine & Fraaije, 2009; Matusinsky *et al.*, 2010). This study suggests that, especially with a lack of resistant cultivars, an integrated management system which reduces reliance on fungicides and hence prolongs their effective life time is required. Further genetic studies using microsatellite markers used at different time points during the growing season and including more populations are needed to give a better insight into the evolutionary forces shaping the genetic structure of this damaging pathogen of barley. Additionally this study indicated the lack of genetic structuring at the field scale and for future experiments a simple sampling design, not taking into account hierarchical sampling, should be sufficient to obtain the necessary information.

Although there has been an increase in studies of *Rcc* epidemiology in recent years, we still lack the critical knowledge of the population biology of the species in many areas. Our indirect population genetic study provides important insight into the biology of the fungus and the evolutionary forces that shape its population structure. However it is important to remember the limitations of this approach, and further direct studies of *Rcc* population biology are required and expected in the coming years.

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Table 1 Final list of ten sets of primers used to amplify SSR loci in populations of *Rcc*.

SSR number	Primers name	Sequence 5'-3'	SSR type	Allele size range in studied populations[a]
1	PentaSSR_1_223nt_F1	GCGTCAAACTGACGAATGAG	$(GGCAT)_5$	228-253
	PentaSSR_1_223nt_R1	ACATCCTTCCAAACACCAGC		
2	PentaSSR_2_233nt_F1	CTCATGTTGCAGAGAGCGAG	$(CTTCA)_5$	245-260
	PentaSSR_2_233nt_R1	AATTTCCGACGTGGATTGAG		
3	PentaSSR_3_253nt_F1	TAGGACAGGAAGACCCGAGA	$(GACAG)_6$	260-275
	PentaSSR_3_253nt_R1	ACCTCGACACCTGAACCTTG		
4	PentaSSR_4_266nt_F1	AGGAGATTAGGGATGCGGTT	$(GTCCT)_5$	285-305
	PentaSSR_4_266nt_R1	TAAATCCATCGAGCCCGTAG		
5	PentaSSR_5_243nt_F1	ATCAACACATCGCGATCAAA	$(CAGCA)_5$	255-275
	PentaSSR_5_243nt_R1	GACAATTGCGGAGTTCCATT		
6	PentaSSR_6_202nt_F1	CTAGGAGAAGAGTGCCGTGG	$(CACAG)_5$	207-217
	PentaSSR_6_202nt_R1	AACACGACGACGATTGTGAA		
7	PentaSSR_7_171nt_F1	GTCCACACATTGACCGAGTG	$(GGTGT)_5$	174-209
	PentaSSR_7_171nt_R1	GGGTTCGCCTGATCTACTGA		
8	PentaSSR_8_131nt_F1	AGATCGCAAAGGATGGATTG	$(GGAAG)_5$	143-148
	PentaSSR_8_131nt_R1	ATTCATTGCACGTTGGTTGA		
11	PentaSSR_11_213nt_F1	TATTCCACACAACGCTCCAA	$(CCTTA)_5$	230-235
	PentaSSR_11_213nt_R1	ATCTCACGCCTCAACGATTC		
12	PentaSSR_12_292nt_F1	ATCAGATCAGCTCCATTGCC	$(ATCCA)_8$	292-347
	PentaSSR_12_292nt_R1	GGTCGGCGATGACATTACTT		
-	M13_For_(-20)_FAM	ACTGTAAAACGACGGCCAGT	-	-

[a] **All sizes comprise M13 primer length**

Table 2 Allelic richness (*A*) and gene diversity (*H*) per locus and population at ten SSR loci in the Czech Republic and the Scottish populations of *Rcc*.

	Czech population (n^c =28)		Scottish population (n =58)	
Loci	*A*	H_{sk}[a]	*A*	H_{sk}
SSR1	3.000	0.474	4.998	0.693
SSR2	3.000	0.442	3.998	0.334
SSR3	3.000	0.659	3.998	0.641
SSR4	4.000	0.585	3.998	0.504
SSR5	1.000	0.000	3.996	0.134
SSR6	2.000	0.138	3.000	0.473
SSR7	4.000	0.373	4.998	0.256
SSR8	2.000	0.071	2.000	0.479
SSR11	2.000	0.349	1.000	0.000
SSR12	8.000	0.844	5.000	0.596
All over the loci (H_t[b])	3.200	0.392	3.699	0.411

[a] ***H_{sk}* =unbiased Nei's (1987) estimator of mean gene diversities for each locus**
[b] ***H_t* =unweighted Nei's (1987) estimator of overall gene diversity**
[c] **n =clone corrected sample size**

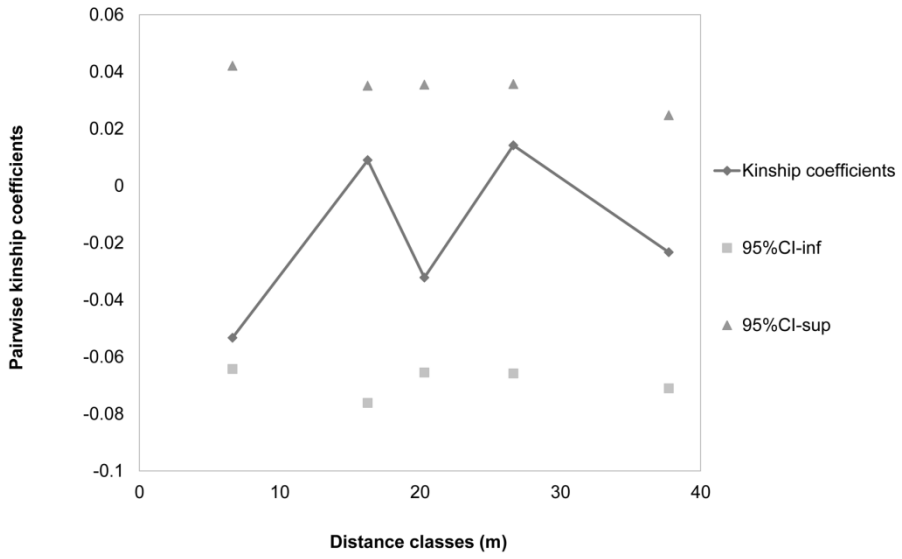
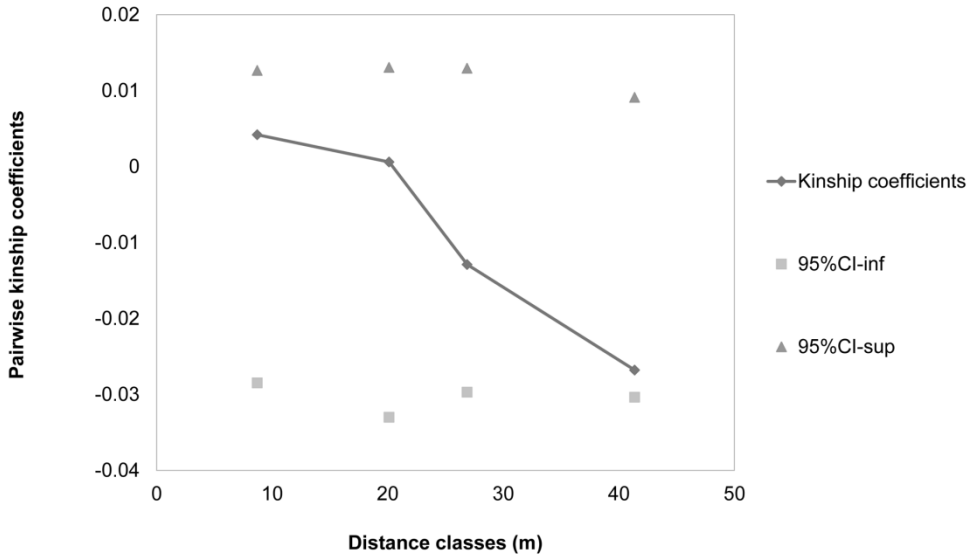


Figure 1 Spatial structure of Czech population divided into five distance classes and plotted against pairwise kinship coefficients according to Loiselle *et al.* (1995), (CI- confidence intervals, inf- inferior, sup- superior).



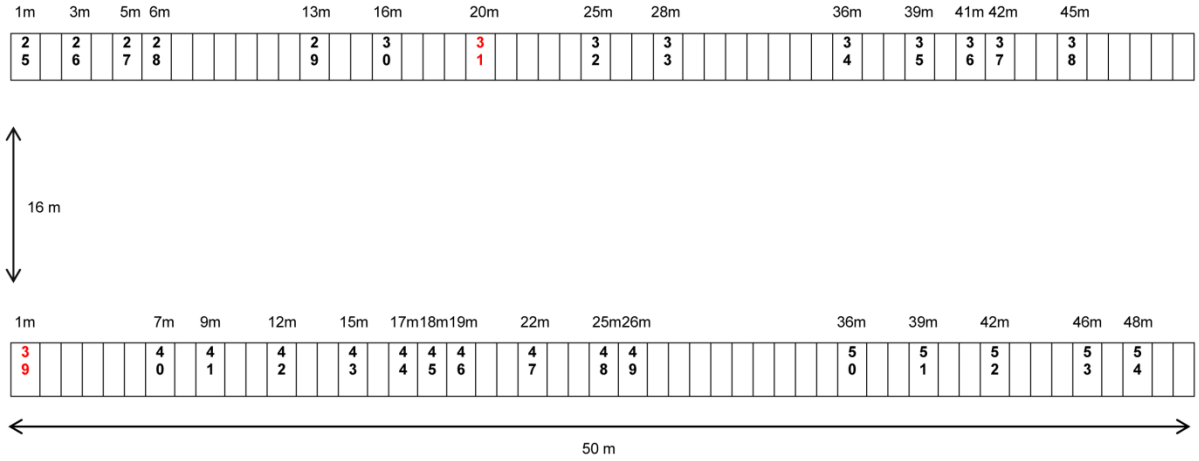
446

447 **Figure 2 Spatial structure of Scottish population divided into four distance classes and plotted**
448 **against pairwise kinship coefficients according to Loiselle *et al.* (1995), (CI- confidence**
449 **intervals, inf- inferior, sup- superior).**

450

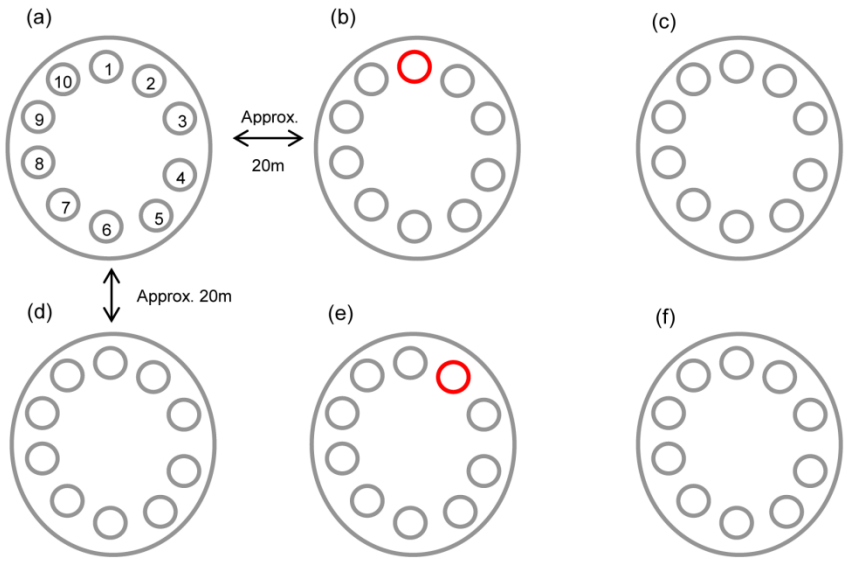
451

452 Supplementary figures



453

454 **Figure A. 1 Sampling strategy in the Czech Republic. Samples were collected in two transect**
455 **lines. In each line 50 isolates, each one meter apart, were sampled. Fourteen leaves from the**
456 **first line and 16 from the second line were used in the study. The numbers in red indicate**
457 **clonal genotypes.**



458

459 **Figure A. 2 Sampling strategy in Scotland. Sixty isolates in two transect lines were amassed. In**
460 **each line three circles and then ten leaf samples around the circle, as indicated by numbers 1-**
461 **10, were collected. Red circles indicate clonal genotypes.**

462

References

Banke S, McDonald BA. 2005. Migration patterns among global populations of the pathogenic fungus *Mycosphaerella graminicola*. *Molecular Ecology*, 14: 1881-1896.

Barnes I, Cortinas MN, Wingfield MJ and Wingfield BD. 2008. Microsatellite markers for the red band needle blight pathogen, *Dothistroma septosporum*. *Molecular Ecology Resources*, 8: 1026-1029.

Barres B, Dutech C, Andrieux A, Halkett F and Frey P. 2012. Exploring the role of asexual multiplication in poplar rust epidemics: impact on diversity and genetic structure. *Molecular Ecology*, 21: 4996-5008.

Barrett LG, Thrall PH, Burdon JJ and Linde CC. 2008. Life history determines genetic structure and evolutionary potential of host-parasite interactions. *Trends in Ecology & Evolution*, 23: 678-685.

Biek R, Real LA. 2010. The landscape genetics of infectious disease emergence and spread. *Molecular Ecology*, 19: 3515-3531.

Brown AHD. 1975. Sample sizes required to detect linkage disequilibrium between two or three loci. *Theoretical Population Biology*, 8: 184-201.

Burt PJ, Rutter J and Ramirez F. 1998. Airborne spore loads and mesoscale dispersal of the fungal pathogens causing Sigatoka diseases in banana and plantain. *Aerobiologia*, 14: 209-214.

Cavara F. 1893. Uber einige parasitische Pilze auf Getreide. *Zeitschrift fur Pflanzenkrankheiten*, 3: 16-26.

Cooke DE, Cano LM, Raffaele S, Bain RA, Cooke LR, Etherington GJ, Deahl KL, Farrer RA, Gilroy EM, Goss EM, Grunwald NJ, Hein I, MacLean D, McNicol JW, Randall E, Oliva RF, Pel MA, Shaw DS, Squires JN, Taylor MC, Vleeshouwers VGAA, Birch PRJ, Lees AK and Kamoun S. 2012. Genome analyses of an aggressive and invasive lineage of the Irish potato famine pathogen. *PLoS Pathogens*, 8: e1002940.

Coombs J, Letcher B and Nislow K. 2008. CREATE: a software to create input files from diploid genotypic data for 52 genetic software programs. *Molecular Ecology Resources*, 8: 578-580.

Dilmaghani A, Gladieux P, Gout L, Giraud T, Brunner PC, Stachowiak A, Balesdent MH and Rouxel T. 2012. Migration patterns and changes in population biology associated with the worldwide spread of the oilseed rape pathogen *Leptosphaeria maculans*. *Molecular Ecology*, 21: 2519-2533.

Fountaine JM, Fraaije BA. 2009. Development of QoI resistant alleles in populations of *Ramularia collo-cygni*. *Aspects of Applied Biology*, 92: 123-126.

Frei P, Gindro K, Richter H and Schuerch S. 2007. Direct-PCR detection and epidemiology of *Ramularia collo-cygni* associated with barley necrotic leaf spots. *Journal of Phytopathology*, 155: 281-288.

Gibson IAS. 1972. Dothistroma blight of *Pinus radiata*. *Annual Review of Phytopathology*, 10: 51-72.

Goudet J. 1995. FSTAT (version 1.2): a computer program to calculate *F*-statistics. *Journal of Heredity*, 86: 485-486.

Goudet J. 2002. FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3.2). Updated from Goudet (1995). Accessed: July/2015. Available from: http://www2.unil.ch/popgen/softwares/fstat.htm.

Gurung S, Goodwin SB, Kabbage M, Bockus WW and Adhikari TB. 2011. Genetic differentiation at microsatellite loci among populations of *Mycosphaerella graminicola* from California, Indiana, Kansas, and North Dakota. *Phytopathology*, 101: 1251-1259.

Gurung S, Short D and Adhikari T. 2013. Global population structure and migration patterns suggest significant population differentiation among isolates of *Pyrenophora tritici-repentis*. *Fungal Genetics and Biology*, 52: 32-41.

Hardy OJ, Vekemans X. 2002. SPAGeDi: a versatile computer program to analyse spatial genetic structure at the individual or population levels. *Molecular Ecology Notes*, 2: 618-620.

Havis ND, Oxley SJP, Piper SR and Langrell SRH. 2006. Rapid nested PCR-based detection of *Ramularia collo-cygni* direct from barley. *FEMS Microbiology Letters*, 256: 217-223.

Havis ND, Oxley SJP and Burnett FJ. 2012. Advances in control of *Ramularia collo-cygni*. In: *Proceedings Crop Protection in Northern Britain 2012,* ed(s). Heilbronn TD, pp. 125-130. Page Bros, Norwich (UK).

527 Havis ND, Nyman M and Oxley SJP. 2014. Evidence for seed transmission and
528 symptomless growth of *Ramularia collo-cygni* in barley (*Hordeum vulgare*).
529 *Plant Pathology*, 63: 727-960.

530 HGCA. 2013. Ramularia leaf spot in barley. Information sheet 21 May 2013.
531 Accessed: Dec/2013. Avaliable from:
532 http://publications.hgca.com/publications/documents/IS21_Ramularia_leaf_sp
533 ot_in_barley.pdf.

534 Hjortshoj R, Ravnshoj A, Nyman M, Orabi J, Backes G, Pinnschmidt H, Havis N,
535 Stougaard J and Stukenbrock E. 2013. High levels of genetic and genotypic
536 diversity in field populations of the barley pathogen *Ramularia collo-cygni*.
537 *European Journal of Plant Pathology*, 136: 51-60.

538 Hollomon DW, Brent KJ. 2009. Combating plant diseases- the Darwin connection.
539 *Pest Management Science*, 65: 1156-1163.

540 Huss H. 2004. The biology of *Ramularia collo-cygni*. In: *Proceedings of the Second*
541 *International Workshop of Barley Leaf Blights- meeting the challenges of*
542 *barley blights,* ed(s). Yahyaoui AH, Brader L, Tekauz A, Wallwork H,
543 Steffenson B, pp. 321-328. ICARDA, Aleppo (Syria).

544 Jarne P, Lagoda PJL. 1996. Microsatellites, from molecules to populations and back.
545 *Trends in Ecology & Evolution*, 11: 424-429.

546 Karadzic D. 1989. *Scirrhia pini* Funk et Parker. Life cycle of the fungus in plantations
547 of *Pinus nigra* Arn. in Serbia. *European Journal of Forest Pathology*, 19: 231-
548 236.

549 Kraj W, Kowalski T. 2013. Microspatial genetic diversity of *Dothistroma septosporum*.
550 *Forest Pathology*, 43: 42-50.

551 Leisova-Svobodova L, Matusinsky P and Kucera L. 2012. Variability of the *Ramularia*
552 *collo-cygni* population in Central Europe. *Journal of Phytopathology*, 160: 701-
553 709.

554 Linde CC, Zhan J and McDonald BA. 2002. Population structure of *Mycosphaerella*
555 *graminicola*: from lesions to continents. *Phytopathology*, 92: 946-955.

556 Linde CC, Zala M and McDonald BA. 2005. Isolation and characterization of
557 microsatellite loci from the barley scald pathogen, *Rhynchosporium secalis*.
558 *Molecular Ecology Notes*, 5: 546-548.

559 Linde CC, Zala M and McDonald BA. 2009. Molecular evidence for recent founder
560 populations and human-mediated migration in the barley scald pathogen
561 *Rhynchosporium secalis*. *Molecular Phylogenetics and Evolution*, 51: 454-464.

562 Loiselle BA, Sork VL, Nason J and Graham C. 1995. Spatial genetic structure of a
563 tropical understory shrub, *Psychotria Officinalis* (Rubiaceae). *American*
564 *Journal of Botany*, 82: 1420-1425.

565 Matusinsky P, Svobodova-Leisova L, Marik P, Tvaruzek L, Stemberkova L,
566 Hanusova M, Minarikova V, Vysohlidova M and Spitzer T. 2010. Frequency of
567 a mutant allele of cytochrome b conferring resistance to QoI fungicides in the
568 Czech population of *Ramularia collo-cygni*. *Journal of Plant Diseases and*
569 *Protection*, 117: 248-252.

570 Matusinsky P, Leisova-Svobodova L, Gubis J, Hudcovicova M, Klcova L, Gubisova
571 M, Marik P, Tvaruzek L and Minarikova V. 2011. Impact of the seed-borne
572 stage of *Ramularia collo-cygni* in barley seed. *Journal of Plant Pathology*, 93:
573 679-689.

574 Matusinsky P, Hanusova M, Stemberkova L, Marik P, Minarikova V, Tvaruzek L,
575 Langer I and Spitzer T. 2013. Response of spring barley cultivars to Ramularia
576 leaf spot in conditions of the Czech Republic. *Cereal Research*
577 *Communications*, 41: 126-132.

578 McDonald BA, Zhan J and Burdon JJ. 1999. Genetic structure of *Rhynchosporium*
579 *secalis* in Australia. *Phytopathology*, 89: 639-645.

580 McDonald BA, Linde C. 2002. Pathogen population genetics, evolutionary potential,
581 and durable resistance. *Annual Review of Phytopathology*, 40: 349-379.

582 Medini M, Hatnza S. 2008. Pathotype and molecular characterization of
583 *Mycosphaerella graminicola* isolates collected from Tunisia, Algeria, and
584 Canada. *Journal of Plant Pathology*, 90: 65-73.

585 Nei M. 1987. *Molecular evolutionary genetics*. Columbia University Press, New York
586 (USA).

587 Oerke EC, Dehne HW. 2004. Safeguarding production- losses in major crops and the
588 role of crop protection. *Crop Protection*, 23: 275-285.

589 Oliveira EJ, Padua JG, Zucchi MI, Vencovsky R and Vieira MLC. 2006. Origin,
590 evolution and genome distribution of microsatellites. *Genetics and Molecular*
591 *Biology*, 29: 294-307.

592 Oxley SJP, Havis ND, Sutherland KG and Nuttall M. 2002. Development of a
593 rationale to identify the causal agent of necrotic lesions in spring barley and to
594 identify control mechanisms. Project report No. 282. HGCA, Kenilworth (UK).

595 Oxley SJP, Havis ND. 2010. Managing *Ramularia collo-cygni* through varietal
596 resistance, seed health and forecasting. Project report No. 463. HGCA,
597 Kenilworth (UK).

598 Peterson GW. 1973. Infection of Austrian and ponderosa pines by *Dothistroma pini* in
599 eastern Nebraska. *Phytopathology*, 63: 1060-1063.

600 Rieux A, De Bellaire L, Zapater MF, Ravigne V and Carlier J. 2013. Recent range
601 expansion and agricultural landscape heterogeneity have only minimal effect
602 on the spatial genetic structure of the plant pathogenic fungus *Mycosphaerella*
603 *fijiensis*. *Heredity*, 110: 29-38.

604 Sachs E, Greil P, Amelung D and Huss H. 1998. *Ramularia collo-cygni*- a
605 rediscovered pathogen of barley in Europe. *Mitteilungen aus der Biologischen*
606 *Bundesanstalt fur Land- und Forstwirtschaft*, 357: 96-97.

607 Sachs E. 2006. The history of research into Ramularia leaf spot on barley.
608 *Nachrichtenblatt Deutscher Pflanzenschutzdienst*, 58: 186-189.

609 Salamati S, Reitan L. 2007. *Ramularia collo-cygni* on spring barley, an overview of its
610 biology and epidemiology. In: *Proceedings of the First European Ramularia*
611 *Workshop- a new disease and challenge in barley production,* ed(s).
612 Koopmann B, Oxley S, Schutzendubel A, von Tiedemann A, pp. 19-35.
613 GOEDOC, Gottingen (Germany).

614 Schlotterer C. 2000. Evolutionary dynamics of microsatellite DNA. *Chromosoma*,
615 109: 365-371.

616 Schuelke M. 2000. An economic method for the fluorescent labeling of PCR
617 fragments. *Nature Biotechnology*, 18: 233-234.

618 Selkoe KA, Toonen RJ. 2006. Microsatellites for ecologists: a practical guide to using
619 and evaluating microsatellite markers. *Ecology Letters*, 9: 615-629.

620 Stefansson TS, Serenius M and Hallsson JH. 2012. The genetic diversity of Icelandic
621 populations of two barley leaf pathogens, *Rhynchosporium commune* and
622 *Pyrenophora teres*. *European Journal of Plant Pathology*, 134: 167-180.

623 Stukenbrock EH, McDonald BA. 2008. The origins of plant pathogens in agro-
624 ecosystems. *Annual Review of Phytopathology*, 46: 75-100.

625 Sunnucks P. 2000. Efficient genetic markers for population biology. *Trends in*
626 *Ecology & Evolution*, 15: 199-203.

627 Thiel T, Michalek W, Varshney R and Graner A. 2003. Exploiting EST databases for
628 the development and characterization of gene-derived SSR-markers in barley
629 (*Hordeum vulgare* L.). *Theoretical and Applied Genetics*, 106: 411-422.

630 Tilman D. 1999. Global environmental impacts of agricultural expansion: the need for
631 sustainable and efficient practices. *Proceedings of the National Academy of*
632 *Sciences*, 96: 5995-6000.

633 Tomsovsky M, Tomesova V, Palovcikova D, Kostovcik M, Rohrer M, Hanacek P and
634 Jankovsky L. 2013. The gene flow and mode of reproduction of *Dothistroma*
635 *septosporum* in the Czech Republic. *Plant Pathology*, 62: 59-68.

636 Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M and Rozen
637 SG. 2012. Primer3- new capabilities and interfaces. *Nucleic Acids Research*,
638 40: e115.

639 Valiere N. 2002. GIMLET: a computer program for analysing genetic individual
640 identification data. *Molecular Ecology Notes*, 2: 377-379.

641 Walters DR, Havis ND and Oxley SJ. 2008. *Ramularia collo-cygni*: the biology of an
642 emerging pathogen of barley. *FEMS Microbiology Letters*, 279: 1-7.

643 Wang XR, Ennos RA, Szmidt AE and Hansson P. 1997. Genetic variability in the
644 canker pathogen fungus, *Gremmeniella abietina*. 2. Fine-scale investigation of
645 the population genetic structure. *Canadian Journal of Botany*, 75: 1460-1469.

646 Weir BS, Cockerham CC. 1984. Estimating *F*-statistics for the analysis of population
647 structure. *Evolution*, 38: 1358-1370.

648 Weir BS. 1996. *Genetic data analysis II*. Sinauer Associates, Inc., Sunderland (USA).

649 Woods JG, Paetkau D, Lewis D, McLellan BN, Proctor M and Strobeck C. 1999.
650 Genetic tagging of free- ranging black and brown bears. *Wildlife Society*
651 *Bulletin*, 27: 616-627.

652 Xhaard C, Fabre B, Andrieux A, Gladieux P, Barres B, Frey P and Halkett F. 2011.
653 The genetic structure of the plant pathogenic fungus *Melampsora larici-*
654 *populina* on its wild host is extensively impacted by host domestication.
655 *Molecular Ecology*, 20: 2739-2755.

656 Zadoks JC, Chang TT and Konzak CF. 1974. Decimal code for growth stages of
657 cereals. *Weed Research*, 14: 415-421.

658 Zaffarano PL, McDonald BA and Linde CC. 2009. Phylogeographical analyses reveal
659 global migration patterns of the barley scald pathogen *Rhynchosporium*
660 *secalis*. *Molecular Ecology*, 18: 279-293.
661
662 *Ramularia collo-cygni* genome browser. Accessed: Jan/2016. Avaliable from:
663 http://ramularia.org/jbrowse.